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Characterization of the phage φ29 protein p5 as a single-stranded DNA binding protein. Function in φ29 DNA–protein p3 replication

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ABSTRACT

The phage φ29 protein p5, required *in vivo* in the elongation step of φ29 DNA replication, was highly purified from *Escherichia coli* cells harbouring a gene 5-containing plasmid and from φ29-infected *Bacillus subtilis*. The protein was characterized as the gene 5 product by amino acid analysis and NH_2-terminal sequence determination. The purified protein p5 was shown to bind to single-stranded DNA and to protect it against nuclease degradation. No effect of protein p5 was observed either on the formation of the p3-dAMP initiation complex or on the rate of elongation. However, protein p5 greatly stimulated φ29 DNA-protein p3 replication at incubation times where the replication in the absence of p5 leveled off.

INTRODUCTION

The *Bacillus subtilis* phage φ29 has a linear, double- stranded DNA 19,285 bp long (1) with the terminal protein p3 covalently linked at the two 5′ ends (2). *In vivo* replication of φ29 starts at either DNA end primed by the terminal protein and proceeds by a strand-displacement mechanism (3–5). Replication of the parental strand being displaced probably starts at the opposite end before complete strand-displacement has occurred, as indicated by the fact that *in vivo* replicative intermediates consisting of double-stranded DNA with two single-stranded tails of different length have been observed by electron microscopy (3).

A purified *in vitro* system able to replicate φ29 DNA has been developed. This system, which requires the terminal protein p3 and the φ29 DNA polymerase p2, can initiate φ29 DNA-protein p3 replication by the formation of the protein p3-dAMP initiation complex catalyzed by the DNA polymerase (6,7), which is able to elongate the initiation complex further to produce full-length φ29 DNA (8). Other viral proteins known to be involved in φ29 DNA replication *in vivo* are the products of genes 1, 5, 6 and 17 (9–11). The function of the gene 1 and 17 products is presently unknown. The viral protein p6 has been purified and shown to interact specifically with the φ29 DNA ends stimulating the formation of the p3-dAMP complex and the transition from initiation to elongation when added to the minimal *in vitro* system containing terminal protein and DNA polymerase (12–14). By *in vivo* shift-up experiments using *ts* 5 mutants, the gene 5 product has been shown to be required in an elongation step of φ29 DNA replication (15). Gene 5 has been recently characterized, cloned under the control of the P_L promoter of phage lambda and protein p5 has been overproduced in *E. coli* (16). In this paper we report the purification of protein p5 and its function in binding to single-stranded DNA. The stimulation of φ29 DNA-protein p3 replication by protein p5 is also shown.

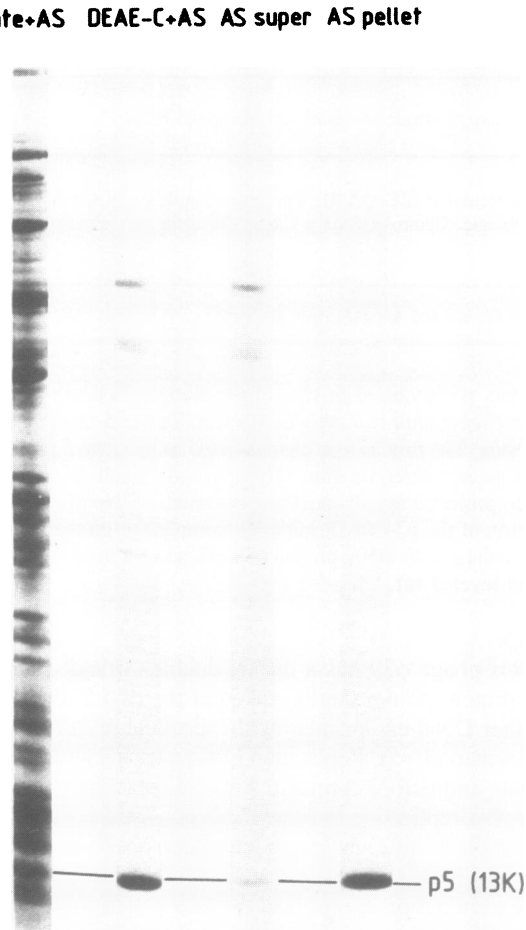

Figure 1. SDS-polyacrylamide gel electrophoresis of purified protein p5. Proteins at various purification steps were subjected to SDS-electrophoresis in slab gels containing a 10–20% acrylamide gradient. After electrophoresis the proteins were stained as indicated in Materials and Methods, section c. Lanes: a) extract precipitated with ammonium sulfate to 65% saturation (80 μg); b) DEAE-cellulose, 50 mM NaCl eluate precipitated with ammonium sulfate to 65% saturation (4.2 μg); c) supernatant from the 1.4 M ammonium sulfate precipitation (2.2 μg); d) 1.4 M ammonium sulfate precipitate (4.1 μg). The position of protein p5 is indicated.

MATERIALS AND METHODS

a) *Bacterial strains, plasmids, phage, proteins and radioactive compounds*

The *E. coli* λ lysogen K-12Δtrp (λN⁻cI857ΔH1) (K-12ΔH1Δtrp) (17) was obtained from M. Zabeau. The gene 5-containing recombinant plasmid pGM26 with gene 5 under the control of the P_L promoter of phage λ was as described (16). The φ29 delayed lysis mutant *sus*14(1242) was as described (18).

Restriction endonucleases, the Klenow fragment of *E. coli* DNA polymerase I and nuclease P1 were from Boehringer Mannheim. The φ29 DNA polymerase p2 and the

Table 1. Summary of protein p5 purification

	Total protein, mg	Protein p5, %	Total protein p5, mg
Extract	720	1.4	10.1
DEAE-cellulose	7.4	47	3.5
AS, 65%	4.2	68	2.9
AS, 1.4 M	2.5	99	2.5

Total protein was determined by the method of Bradford (24). SDS-polyacrylamide gel electrophoresis was carried out at the different purification steps to follow the presence of protein p5. The % of protein p5 was determined by densitometry of the stained bands.

terminal protein p3 were purified as described (6,19). [α-^{32}P]dATP ($\sim$400 Ci/mmol) and [γ-^{32}P] ATP (3000 Ci/mmol) were from Amersham International plc.

b) *Preparation and labelling of nucleic acids*

Proteinase K-treated ϕ29 DNA and ϕ29 DNA-protein p3 complex were isolated as described (20,21). The ϕ29 DNA HindIII L fragment, 273 bp long, from the right ϕ29 DNA end, was isolated by electrophoresis on 3.5% polyacrylamide gels in 0.1 M Tris-borate, pH 8.3, 2 mM EDTA (TBE) and labelled by filling-in with the Klenow enzyme. A 253 nucleotide long single-stranded DNA fragment from an internal region of ϕ29 DNA, from nucleotide 11315 to 11568 (1), labelled at the 5′ end with polynucleotide kinase and [γ-^{32}P]ATP, was a gift from I. Barthelemy. ^{3}H-labelled M13-DNA was prepared by *in vivo* labelling with ^{3}H-thymidine and isolation from purified phage particles.

c) *Purification of protein p5*

Ten g of *E. coli* K12ΔH1Δtrp cells harbouring the gene 5-containing recombinant plasmid pGM26, induced for 2.5 h at 42°C, were ground with alumina (20 g) and extracted with buffer A (50 mM Tris-HCl, pH 7.5, 5% glycerol) containing 0.3 M KCl. The lysate was centrifuged for 10 min at 16,500×g and the pellet re-extracted with the same buffer. The two supernatants were pooled and precipitated with ammonium sulfate to 65% saturation. The pellet was dissolved in buffer A, dialyzed against the same buffer, diluted with buffer A + 20% glycerol and passed through a DEAE-cellulose column (2.7 cm×10 cm) equilibrated with buffer A + 10 mM NaCl. The column was washed first with buffer A + 20% glycerol, then with buffer A, and protein p5 was finally eluted with buffer A + 50 mM NaCl. The fractions containing protein p5 were pooled and precipitated with ammonium sulfate to 65% saturation. The pellet was resuspended in 1.2 ml of buffer A + 1.4 M ammonium sulfate and 50% glycerol. The pellet remaining after centrifugation, containing most of protein p5, was dissolved in buffer A + 50% glycerol. Protein p5 was purified by a similar procedure from *B. subtilis* cells infected with the ϕ29 delayed lysis mutant *sus*14(1242). In all purification steps protein p5 was followed by SDS-polyacrylamide gel electrophoresis.

When indicated, after the last purification step, protein p5 was centrifuged for 24 h at 260,000×g at 0°C in a 5 ml 15 to 30% (v/v) glycerol gradient in 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl. After centrifugation, 0.2 ml fractions were collected and the presence of protein p5 was determined as indicated above.

Electrophoresis of proteins was carried out in slab gels containing 10−20% acrylamide gradients in the presence of 0.1% SDS (22). After electrophoresis the proteins were stained as described by Fairbanks et al. (23). Protein concentration was determined by the method of Bradford (24).

Table 2. Amino acid composition of purified protein p5

Residue	Predicted[a]	Observed[b]
Asp + Asn	13	12.1
Thr	14	13.6
Ser	10	9.8
Glu + Gln	15	14.8
Pro	1	1.1
Gly	9	9.4
Ala	12	12.1
Val	9	9.0
Met	1	0.8
Ile	9	8.6
Leu	9	9.2
Tyr	3	2.4
Phe	7	6.9
Trp	0	N.D.
Lys	10	10.0
His	1	1.1
Arg	1	0.9

[a]Amino acid composition predicted from the nucleotide sequence (29). The amino-terminal Met has been included.
[b]Amino acid composition of purified protein p5. Each value represents the average after 24, 48 and 72 h hydrolysis, except the values for Thr and Ser that were extrapolated to zero hours of hydrolysis. Values represent residues per molecule. Trp was not determined (N.D.).

d) *Amino acid analysis and protein sequence determination*
Purified protein p5 was hydrolyzed with 100 μl of 5.7 M HCl containing 0.05% (v/v) 2-mercaptoethanol in evacuated and sealed tubes at 110°C for 24, 48 and 72 h. The analysis were performed on a Beckman 121-M analyzer equipped with a Beckman integrator 126 data system.

The NH_2-end of purified protein p5 was sequenced in a Beckman sequencer (model 890 D) according to the method of Edman and Begg (25). The sequence of the protein was determined using the Beckman protein/peptide/micro/macrosequencing program as described (26).

e) *Gel retardation assay*
Protein p5 in the amount indicated in each case was incubated for 30 min at 4°C in a final volume of 20 μl of a buffer containing 12 mM Tris-HCl, pH 7.5 and 1 mM EDTA with ^{32}P-labelled HindIII L fragment, 273 bp long, from the right end of φ29 DNA or with the ^{32}P-labelled single-stranded internal DNA fragment, 253 nucleotides long, which was described above. Protein p5-DNA complexes were resolved in low ionic strength polyacrylamide gels essentially as described (27). After electrophoresis the gels were dried and autoradiographed.

f) *Assay for the formation of the p3-dAMP initiation complex*
The incubation mixture for the initiation reaction contained, in 20 μl, 50 mM Tris-acetate, pH 7.5, 10 mM Mg acetate, 1 mM dithiothreitol, 10 mM $(NH_4)_2SO_4$, 20 mM NaCl, 25 μg of bovine serum albumin, φ29 DNA-protein p3 (0.22 μg), 0.25 or 40 μM [α-^{32}P] dATP (2 μCi) and purified proteins p2 (24 ng) and p3 (15 ng), in the absence or presence of purified protein p5. When indicated, elongation was allowed to occur in the presence

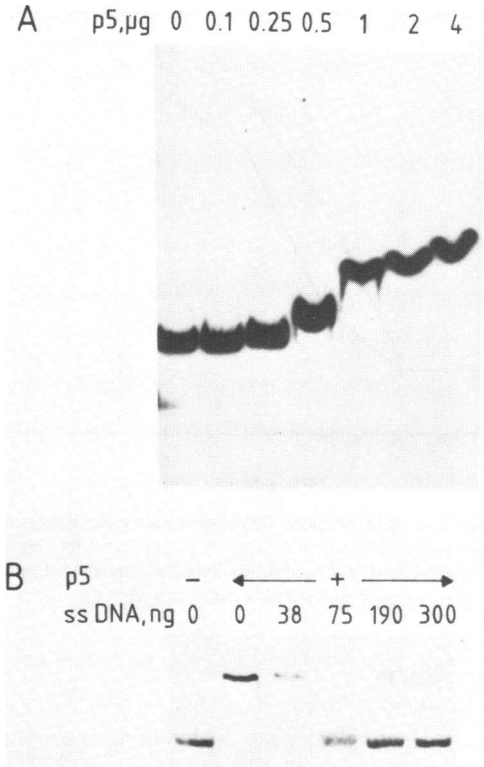

Figure 2. Binding of protein p5 to single-stranded DNA. A ^{32}P-labelled single-stranded DNA fragment 253 nucleotides long (10 ng) was incubated with the indicated amounts of purified protein p5 (A) or with 1 μg of protein p5 in the presence of the indicated amounts of non-radioactive, heat-denatured HindIII L fragment, 273 nucleotides long (B). The protein-DNA complexes were separated by polyacrylamide gel electrophoresis as indicated in Materials and Methods, section e. After electrophoresis the gels were dried and autoradiographed.

of 40 μM each dCTP, dGTP, dTTP and [α-^{32}P]dATP (2 μCi) in the absence or presence of protein p5. After the indicated times at 30°C the reaction was stopped and the samples were treated with micrococcal nuclease and subjected to SDS-polyacrylamide gel electrophoresis as described (13).

g) *Replication assay*

The incubation mixture contained, in 20 μl, 50 mM Tris-acetate, pH 7.5, 10 mM Mg acetate, 1 mM dithiothreitol, 10 mM $(NH_4)_2SO_4$, φ29 DNA-protein p3 (0.22 μg), 40 μM each dCTP, dGTP, dTTP and [α-^{32}P] dATP (2 μCi), bovine serum albumin (25 μg) and purified proteins p2 (24 ng) and p3 (15 ng). When indicated, purified protein p5 was added. After the indicated times at 30°C the reaction was stopped and the samples were filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS. The Cerenkov radiation of the excluded fraction was counted. When the rate of elongation was to be determined, the labelled DNA from the excluded fraction was treated with proteinase K and subjected to alkaline agarose gel electrophoresis (28).

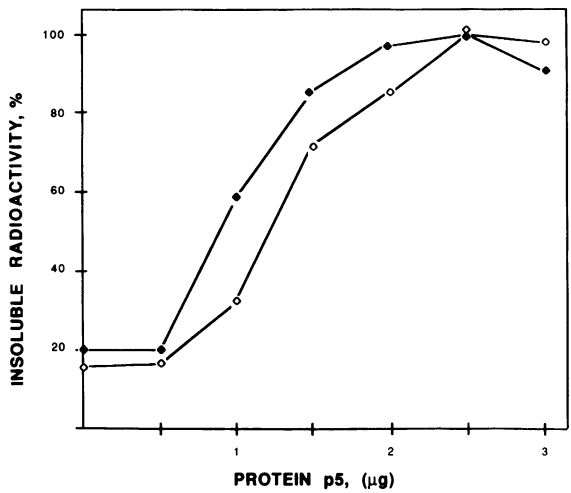

Figure 3. Protection by protein p5 of single-stranded DNA against nuclease degradation. ^{3}H-labelled M13-DNA (◆, 20 ng or ◇, 100 ng) was incubated in a final volume of 20 μl of a buffer containing 30 mM Tris-acetate, pH 7.5, 10 mM Mg acetate and 0.1 mM $ZnCl_2$ with the indicated amounts of purified protein p5 for 3 min at 30°C. Then, nuclease P1 (0.4 μg) was added and, after 5 min at 30°C, the trichloroacetic acid-insoluble radioactivity was determined.

RESULTS AND DISCUSSION

1. *Purification of protein p5*

E. coli K12ΔH1Δtrp cells carrying the gene 5-containing recombinant plasmid pGM26 or *B. subtilis* cells infected with the phage φ29 mutant *sus*14(1242) were used as a source of protein p5 for purification. The protein p5 present in the *E. coli* extracts amounted to ~1.4% of the total protein after 2.5 h of induction at 42°C and that present in the *B. subtilis* extracts was ~2.7% of the total protein. The extracts, after precipitation with ammonium sulfate, were purified by DEAE-cellulose chromatography; most of protein p5 was eluted with 50 mM NaCl. After concentration with ammonium sulfate to 65% saturation, protein p5 was selectively precipitated with 1.4 M ammonium sulfate. Figure 1 shows the analysis by SDS-polyacrylamide gel electrophoresis of the different fractions of the purification from the *E. coli* cells. Densitometric analysis of the protein at the final step indicated that p5 was about 99% pure. Table 1 shows a summary of the purification. About 2.5 mg and 4 mg of purified protein p5 were obtained from 10 g of *E. coli* and *B. subtilis* cells, respectively.

2. *Amino acid analysis of purified protein p5*

Table 2 shows that the amino acid analysis of protein p5 purified from *E. coli* cells gives values very similar to those deduced from the DNA sequence of gene 5 (29). Moreover, the NH_2- terminal sequence of the purified protein p5 showed the sequence Met-Glu-Asn-Thr-Asn-Ile-Val, in agreement with the one predicted from the nucleotide sequence.

3. *Protein p5 binds to single-stranded DNA and protects it against nuclease degradation*

When the binding of protein p5 to the double-stranded HindIII L fragment, 273 bp long, was studied by a gel retardation assay, only a very small amount of DNA was shifted with up to 4 μg of protein (not shown). However, as shown in Fig. 2A, when a 253 nucleotide long single-stranded DNA fragment was used, some retardation of the band

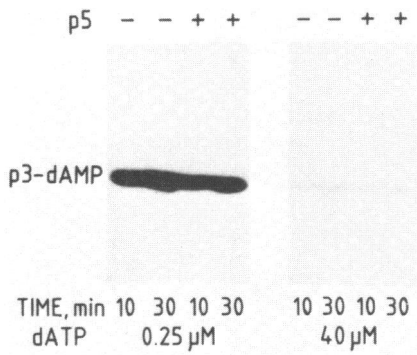

Figure 4. Effect of protein p5 on the formation of the p3-dAMP initiation complex. ϕ29 DNA-protein p3 complex was incubated with purified proteins p2 and p3 and either 0.25 or 40 μM [α-^{32}P]dATP (2 μCi) as indicated in Materials and Methods, section f, in the absence or presence of purified protein p5 (9 μg). After incubation for the indicated times at 30°C the samples were processed and subjected to SDS-polyacrylamide gel electrophoresis as described in Materials and Methods, section f.

was seen even at 0.25 μg of protein p5, the band shift being increased with amounts of up to 4 μg of protein p5. The binding of 1 μg of protein p5 to the single-stranded DNA fragment was resistant to 120 mM NaCl, some inhibition being observed at 160 mM NaCl (not shown). On the other hand, as shown in Fig. 2B, most of the binding of 1 μg of protein p5 to 10 ng of the single-stranded DNA fragment was competed for with 75 ng of non-radioactive heat-denatured HindIII L fragment. The mechanism of interaction of protein p5 with single-stranded DNA is being studied further. In addition, Fig. 3 shows that binding of protein p5 to single-stranded DNA protects the latter against nuclease P1 degradation. These results suggest that a possible role of protein p5 in the elongation stage of ϕ29 DNA replication *in vivo* could be the protection of the single-strand that is being displaced against nuclease degradation (see later). Consistent with such a role in ϕ29 DNA replication is the fact that p5 is a very abundant protein in ϕ29-infected *B. subtilis* cells (~700,000 molecules per cell).

4. *Stimulation of ϕ29 DNA-protein p3 replication by protein p5*

Fig. 4A shows that protein p5 up to 9 μg had no effect on the *in vitro* formation of the protein p3-dAMP initiation complex (which can occur at low dATP concentration) or on elongation of the complex to p3-$(dAMP)_2$ and p3-$(dAMP)_3$ which takes place at a higher dATP concentration, as it was expected from the *in vivo* results which indicated a role of protein p5 in the elongation of replication (15). When the effect of protein p5 on ϕ29 DNA-protein p3 replication was tested, only a small stimulation was obtained at incubation times of 10 min or shorter and no effect on the rate of elongation was observed (Fig. 5A, inset). At longer incubation times the stimulation by protein p5 greatly increased; whereas the replication of the control without protein p5 leveled off, in its presence the replication continued essentially in a linear way for over 40 min (Fig. 5A). As a control, when protein p5 was heated for 5 min at 90°C, no stimulation of replication was observed. The net DNA mass synthesized after 40 min of incubation in the absence of protein p5 was 80 ng. In the presence of protein p5 the net DNA mass synthesized was 390 ng indicating that approximately two rounds of replication occurred on average. Analysis by alkaline agarose gel electrophoresis of the DNA synthesized in the presence of protein p5 showed the presence of full-length ϕ29 DNA as the major product after 7 min of

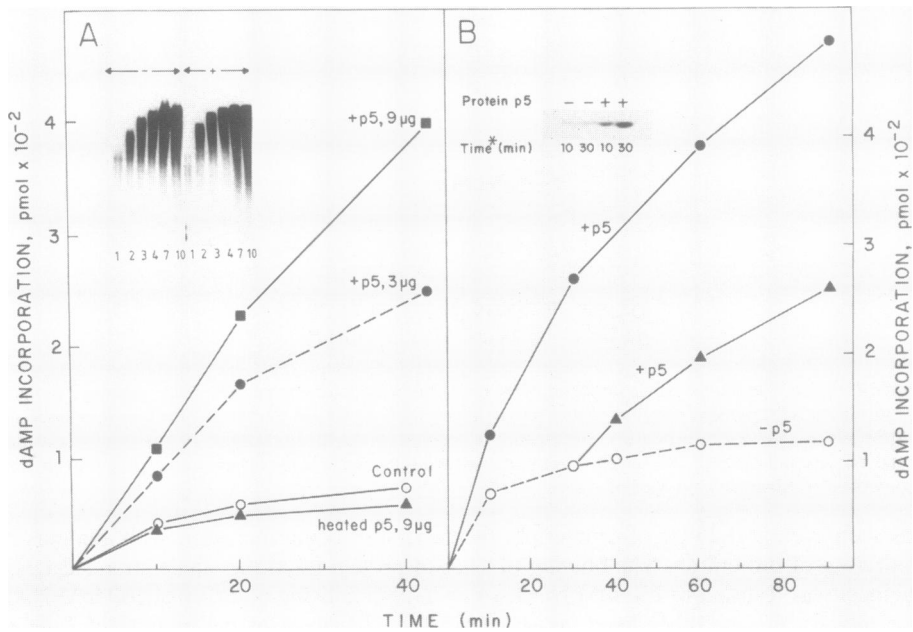

Figure 5. Effect of protein p5 on ϕ29 DNA-protein p3 replication. A. ϕ29 DNA-protein p3 complex was incubated with purified proteins p2 and p3 in the presence of 40 μM each dCTP, dGTP, dTTP and [α-^{32}P]dATP (2 μCi) as indicated in Materials and Methods, section g, in the absence or presence of the indicated amounts of purified protein p5. The samples taken at the times in min indicated in the inset were subjected to alkaline agarose gel electrophoresis. − and + indicates the absence or presence of protein p5. After electrophoresis the gels were dried and autoradiographed. B. As in A, except that 9 μg of protein p5 were added at zero time or after 30 min at 30°C when indicated. After incubation for the indicated times at 30°C the samples were processed and the incorporated radioactivity counted as described in Materials and Methods, section g. At the times indicated in the inset after 30 min of incubation without protein p5 (time*) half of the samples in B were treated with micrococcal nuclease and subjected to SDS-polyacrylamide gel electrophoresis as described in Materials and Methods, section f.

incubation (Fig. 5A, inset and not shown). In addition, as shown in Fig. 5B, the stimulation by protein p5 added at 30 min after replication had started, when the DNA synthesis essentially leveled off, was similar to that obtained when protein p5 was present from the beginning. It can be also seen that the replication proceeded in a linear way for at least 90 min in the presence of protein p5 added either at zero time or after 30 min of incubation. When the amount of the p3-dAMP complex resulting from micrococcal nuclease degradation of the elongation products was determined in the samples elongated in the absence of protein p5 or in the presence of the protein, added after 30 min of incubation, stimulation by protein p5 could be seen (Fig. 5B, inset). These results suggest that protein p5 stimulates ϕ29 DNA replication because it allows reinitiations to occur, possibly by overcoming inhibition by the increasing amounts of single-stranded DNA produced during replication. The stimulation by protein p5 was also seen in the presence of the viral protein p6 in an additive way (results not shown), suggesting that the two proteins act independently during *in vitro* ϕ29 DNA-protein p3 replication.

To analyze the effect of protein p5 on the production of single-stranded DNA, ϕ29 DNA-protein p3 was treated with the restriction nuclease ClaI that produces two fragments, 6147

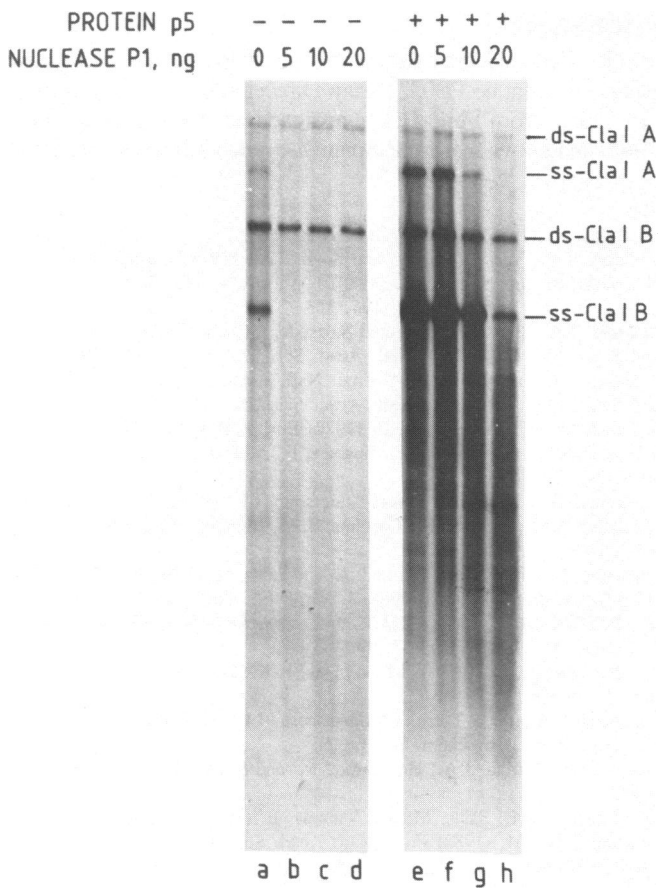

Figure 6. Protection by protein p5 against nuclease P1 in the replication of ClaI fragments of ϕ/29 DNA-protein p3. ϕ/29 DNA-protein p3 complex treated with ClaI was incubated with purified proteins p2 and p3 in the presence of 40 μM each dCTP, dGTP, dTTP and [α-^{32}P]dATP (1 μCi) as indicated in Materials and Methods, section g, in the absence or presence of purified protein p5 (9 μg), without (lanes a and e) or with (lanes b–d and f–h) 0.2 mM $ZnCl_2$ and nuclease P1 as indicated. After incubation for 40 min at 30°C the samples were subjected to electrophoresis in 0.7% agarose gels in TBE. After electrophoresis the gels were dried and autoradiographed.

and 13138 bp long, with protein p3 present at only one end. Fig. 6 shows that the amount of displaced single-strand was greatly increased in the presence of protein p5 (lanes a and g) in agreement with the above results on the reinitiations that occur in the presence of protein p5. In addition, the presence of protein p5 protected the single-stranded DNA against degradation by nuclease P1 (Fig. 6, lanes b–d and f–h). These results suggest that a role of protein p5 *in vivo* could be to protect the single- stranded DNA against nuclease degradation. In addition, the finding that protein p5 prevents the cessation of ϕ29 DNA-protein p3 replication at long incubation times suggests that the protein, by binding to the single-stranded DNA produced, may maintain it in extended form and, thus, avoid inhibition. Analysis by electron microscopy of ϕ29 DNA replication in the presence of protein p5 supports this possibility (J.M. Sogo, G.M. and M.S., unpublished results).

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